Pharmacogenomics: Translating functional genomics into rational therapeutics

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Abstract:
Genetic polymorphisms in drug-metabolizing enzymes, transporters, receptors and other drug targets have been linked to interindividual differences in the efficacy and toxicity of many medications.

Full Text:

[Headnote]
Genetic polymorphisms in drug-metabolizing enzymes, transporters, receptors, and other drug targets have been linked to interindividual differences in the efficacy and toxicity of many medications. Pharmacogenomic studies are rapidly elucidating the inherited nature of these differences in drug disposition and effects, thereby enhancing drug discovery and providing a stronger scientific basis for optimizing drug therapy on the basis of each patient's genetic constitution.

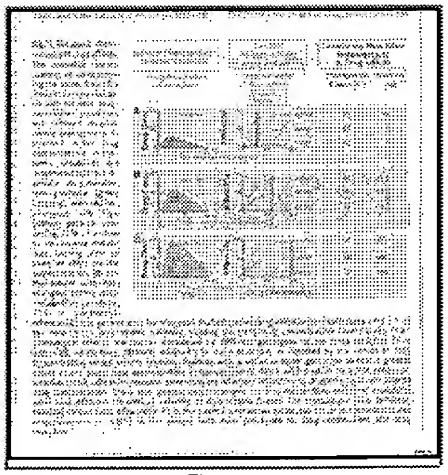

Fig. 1.

There is great heterogeneity in the way individuals respond to medications, in terms of both host toxicity and treatment efficacy. Potential causes for such variability in drug effects include the pathogenesis and severity of the disease being treated; drug interactions; and the individual's age, nutritional status, renal and liver function, and concomitant illnesses. Despite the potential importance of these clinical variables in determining drug effects, it is now recognized that inherited differences in the metabolism and disposition of drugs, and genetic polymorphisms in the targets of drug therapy (such as receptors), can have an even greater influence on the efficacy and toxicity of medications. Clinical observations of such inherited differences in drug effects were first documented in the 1950s, exemplified by the relation between prolonged muscle relaxation after suxamethonium and an inherited deficiency of plasma cholinesterase (1), hemolysis after antimalarial therapy and the inherited level of erythrocyte glucose

6-phosphate dehydrogenase activity (2), and peripheral neuropathy of isoniazid and inherited differences in acetylation of this medication (3). Such observations gave rise to the field of "pharmacogenetics," which focuses largely on genetic polymorphisms in drug-metabolizing enzymes and how this translates into inherited differences in drug effects [reviewed in (4)].

The molecular genetic basis for these inherited traits began to be elucidated in the late 1980s, with the initial cloning and characterization of a polymorphic human gene encoding the drug-metabolizing enzyme debrisoquin hydroxylase (CYP2D6) (5). Genes are considered functionally "polymorphic" when allelic variants exist stably in the population, one or more of which alters the activity of the encoded protein in relation to the wild-type sequence. In many cases, the genetic polymorphism is associated with reduced activity of the encoded protein, but there are also examples where the allelic variant encodes proteins with enhanced activity. Since the cloning and characterization of CYP2D6, human genes involved in many such pharmacogenetic traits have been isolated, their molecular mechanisms have been elucidated, and their clinical importance has been more clearly defined. Inherited differences in drug-metabolizing capacity are generally monogenic traits, and their influence on the pharmacokinetics and pharmacologic effects of medications is determined by their importance for the activation or inactivation of drug substrates. The effects can be profound toxicity for medications that have a narrow therapeutic index and are inactivated by a polymorphic enzyme (for example, mercaptopurine, azathioprine, thioguanine, and fluorouracil) (6) or reduced efficacy of medications that require activation by an enzyme exhibiting genetic polymorphism (such as codeine) (7).

However, the overall pharmacologic ef fects of medications are typically not monogenic traits; rather, they are determined by the interplay of several genes encoding proteins involved in multiple pathways of drug metabolism, disposition, and effects. The potential polygenic nature of drug response is illustrated in Fig. 1, which depicts the hypothetical effects of two polymorphic genes: one that determines the extent of drug inactivation and another that determines the sensitivity of the drug receptor. The polymorphic drug-metabolizing enzyme, which exhibits codominant inheritance (that is, three phenotypes), determines the plasma concentrations to which each individual is exposed, whereas the polymorphic receptor determines the nature of response at any given drug concentration. This example assumes that drug toxicity (Fig. 1, red lines) is determined by nonspecific effects or through receptors that do not exhibit functionally important genetic polymorphisms, although clearly toxicity can also be determined by genetic polymorphisms in drug receptors. Thus, the individual with homozygous wild-type drug-metabolizing enzymes and drug receptors (Fig. 1A) would have a high probability of therapeutic efficacy and a low probability of toxicity, in contrast to an individual with homozygous mutant genotypes for the drug-metabolizing enzyme and the drug receptor, in which the likelihood of efficacy is low and that of toxicity is high (Fig. IC).

Such polygenic traits are more difficult to elucidate in clinical studies, especially when a medication's metabolic fate and mechanisms of action are poorly defined. However, biomedical research is rapidly defining the molecular mechanisms of pharmacologic ef fects, genetic determinants of disease pathogenesis, and functionally important polymorphisms in genes that govern drug metabolism and disposition. Moreover, the Human Genome Project, coupled with functional genomics and high-throughput screening methods, is providing powerful new tools for elucidating polygenic components of human health and disease. This has spawned the field of "pharmacogenomics", which aims to capitalize on these insights to discover new therapeutic targets and interventions and to elucidate the constellation of genes that determine the efficacy and toxicity of specific medications. In this context, pharmacogenomics refers to the entire spectrum of genes that determine drug behavior and sensitivity, whereas pharmacogenetics is often used to define the more narrow spectrum of inherited differences in drug metabolism and disposition, although this distinction is arbitrary and the two terms are now commonly used interchangeably. Ultimately, knowledge of the genetic basis for drug disposition and

response should make it possible to select many medications and their dosages on the basis of each patient's inherited ability to metabolize, eliminate, and respond to specific drugs. Herein, we provide examples that illustrate the current status of such pharmacogenomic research and discuss the prospects for near-term advances in this field.

Genetic Potymorphisms in Drug Metabolism and Disposition

Until recently, clinically important genetic polymorphisms in drug metabolism and disposition were typically discovered on the basis of phenotypic differences among individuals in the population (8), but the framework for discovery of pharmacogenetic traits is rapidly changing. With recent advances in molecular sequencing technology, gene polymorphisms [such as single-nucleotide polymorphisms (SNPs), and especially SNPs that occur in gene regulatory or coding regions (cSNPs)] may be the initiating discoveries, followed by biochemical and, ultimately, clinical studies to assess whether these genomic polymorphisms have phenotypic consequences in patients. This latter framework may permit the elucidation of polymorphisms in drug-metabolizing enzymes that have more subtle, yet clinically important consequences for interindividual variability in drug response. Such polymorphisms may or may not have clear clinical importance for affected medications, depending on the molecular basis of the polymorphism, the expression of other drug-metabolizing enzymes in the patient, the presence of concurrent medications or illnesses, and other polygenic clinical features that impact upon drug response. In Fig. 2, we have highlighted those drug-metabolizing enzymes known to exhibit genetic polymorphisms with incontrovertible clinical consequences; however, almost every gene involved in drug metabolism is subject to common genetic polymorphisms that may contribute to interindividual variability in drug response. Table 1 provides examples of how these genetic polymorphisms can translate into clinically relevant inherited differences in drug disposition and effects, a comprehensive summary of which is available at www.sciencemag.org/feature/data/1044449. shl.

All pharmacogenetic polymorphisms studied to date differ in frequency among ethnic and racial groups. In fact, the slow acetylator phenotype was originally suspected to be genetically determined because of the difference in frequency of isoniazid-induced neuropathies observed in Japan versus those observed in the United States (9). The marked racial and ethnic diversity in the frequency of functional polymorphisms in drug- and xenobiotic-metabolizing enzymes dictates that race be considered in studies aimed at discovering whether specific genotypes or phenotypes are associated with disease risk or drug toxicity.

It is now well recognized that adverse drug reactions may be caused by specific drug-metabolizer phenotypes. This is illustrated by the severe and potentially fatal hematopoietic toxicity that occurs when thiopurine methyltransferase-deficient patients are treated with standard does of azathioprine or mercaptopurine (6). Another example is the slow acetylator phenotype that has been associated with hydralazine-induced lupus, isoniazid-induced neuropathies, dye-associated bladder cancer, and sulfonamide-induced hypersensitivity reactions (9, 10); in all cases, acetylation of a parent drug or an active metabolite is an inactivating pathway. N Acetyltransferase is an enzyme that conjugates substrates with a more water-soluble small molecular moiety. Such conjugation reactions are frequently, but not always, detoxifying, in that they often "mask" a more reactive functional group and usually enhance urinary or biliary excretion of substrates. There are many examples in which the combination of a genetic defect in a conjugation pathway (Fig. 2, right), coupled with a wild-type phenotype for an oxidation pathway (Fig. 2, left), many of which can make substrates more reactive through the insertion of oxygen or other chemical modifications, results in a phenotype particularly predisposed to adverse effects from a medication or environmental substance. Alternatively, increased CYP IA activity (an enzyme catalyzing a phase I oxidation reaction), coupled with slow acetylation (a phase II conjugation reaction), resulted in less myelosuppression from the active metabolites of the anticancer agent amonafide (11). Because every

individual represents a combination of drug-metabolizer phenotypes, given the large number of enzymes involved in drug metabolism, it is apparent that some individuals are destined to have unusual reactions to drugs or to combinations of drugs due to the coincident occurrence of multiple genetic defects in drugmetabolizing enzymes. Such an alignment of genotypes, particularly when coupled with polymorphisms in drug receptors, is likely to constitute part of the mechanism for so-called "idiosyncratic" drug reactions.

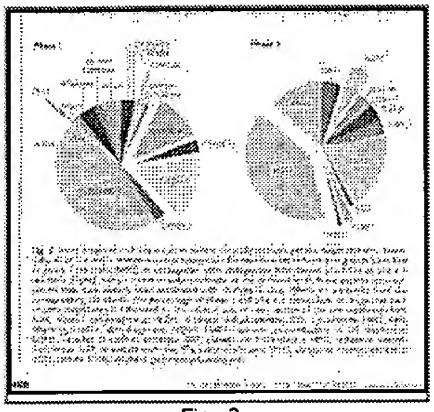

Fig. 2.

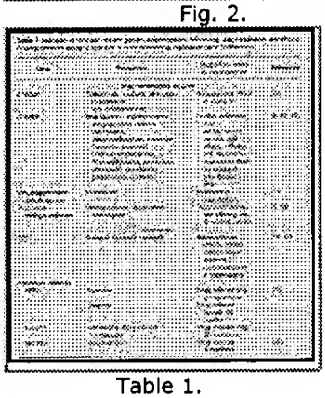

Table 1.

In addition to detoxifying and eliminating drugs and metabolites, drug-metabolizing enzymes are often required for activation of prodrugs. Many opioid analgesics are activated by CYP2D6 (7), rendering the 2 to 10% of the population who are homozygous for nonfunctional CYP2D6 mutant alleles relatively resistant to opioid analgesic effects. It is thus not surprising that there is remarkable interindividual variability in the adequacy of pain relief when uniform doses of codeine are widely prescribed.

For many genetic polymorphisms of drugmetabolizing enzymes, there is no evident phenotype in the absence of a drug challenge, perhaps because these enzymes are not critical for metabolism of endogenous compounds in physiologically essential pathways. However, some drug-metabolism genotypes may result in a phenotype in the absence of drug; for example, it has been postulated that CYP2D6-poor metabolizers are less pain tolerant than extensive metabolizers because of a defect in synthesizing endogenous morphine (12) and that certain forms of dihydropyrimidine dehydrogenase deficiency are associated with mental retardation (13). Moreover, the risk of some cancers has been linked to polymorphisms in drug-metabolizing enzymes, which may be due to an impaired ability to inactivate exogenous or endogenous mutagenic molecules.

As depicted in Fig. 2, CYP3A4 is the human enzyme known to be involved in the metabolism of the largest number of medications. Thus far, no completely inactivating mutations have been discovered in the human CYP3A4 gene, although a common polymorphism in the CYP3A4 promoter has been recently described (14). For enzymes that apparently do not have critical endogenous substrates (for example,

CYP2C19, CYP2D6, and TPMT), the molecular mechanisms of inactivation include splice site mutations resulting in exon skipping (for example, CYP2C19), microsatellite nucleotide repeats (for example, CYP2D6), gene duplication (for example, CYP2D6), point mutations resulting in early stop codons (for example, CYP2D6), amino acid substitutions that alter protein stability or catalytic activity (for example, TPMT, NA T2, CYP2D6, CYP2Cl9, and CYP2C9), or complete gene deletions (for example, GSTM1 and CYP2D6). It is remarkable that even for rare phenotypes such as thiopurine methyltransferase deficiency (which occurs in only 1 in 300 individuals), a small number of recurring mutations have been shown to account for most of the mutant alleles in humans (6). For this and other drug-metabolizing genes, the frequency of SNPs and other genetic defects appears to be more common than the frequency of "1 per 1000 base pairs" that is cited for the human genome. Perhaps it is because some "drug"-metabolizing enzymes are dispensable or redundant with other enzymes (such as CYP2D6 and CYP2C 19) that genetic polymorphisms of drug-metabolizing enzymes are so common.

Genetic Polymorphisms in Drug Transporters

Although passive diffusion accounts for cellular uptake of some drugs and metabolites, increased emphasis (15) is being placed on the role of membrane transporters in absorption of oral medications across the gastrointestinal tract; excretion into the bile and urine; distribution into "therapeutic sanctuaries," such as the brain and testes; and transport into sites of action, such as cardiovascular tissue, tumor cells, and infectious microorganisms. It has been proposed that some of these transporters, such as P-glycoprotein, may not be essential for viability, because knockout mice appear normal until challenged with xenobiotics. However, other transporters are likely to play critical roles in transport of endogenous substances. Although polymorphisms in P-glycoprotein have been reported (16), and such variation may have functional importance for drug absorption and elimination, the clinical relevance of polymorphisms in drug transporters has not yet been fully elucidated.

Genetic Polymorphisms in Drug Targets

Most drugs interact with specific target proteins to exert their pharmacological effects, such as receptors, enzymes, or proteins involved in signal transduction, cell cycle control, or many other cellular events. Molecular studies have revealed that many of the genes encoding these drug targets exhibit genetic polymorphism; which in many cases alters their sensitivity to specific medications. Such examples include polymorphisms in beta-adrenergic receptors and their sensitivity to P-agonists in asthmatics (17), angiotensin converting enzyme (ACE) and its sensitivity to ACE inhibitors (18), angiotensin II Tl receptor and vascular reactivity to phenylephrine (19) or response to ACE inhibitors (20), sulfonylurea receptor and responsiveness to sulfonylurea hypoglycemic agents (21), and 5-hydroxtryptamine receptor and response to neuroleptics such as clozapine (22). In addition, genetic polymorphisms that underlie disease pathogenesis can also be major determinants of drug efficacy, such as mutations in the apolipoprotein E gene and responsiveness of patients with Alzheimer's disease to tacrine therapy (23) or cholesteryl ester transfer protein polymorphisms and efficacy of pravastatin therapy in patients with coronary atherosclerosis (24). Finally, the risk of adverse drug effects has been linked to genetic polymorphisms that predispose to toxicity, such as dopamine D3 receptor polymorphism and the risk of drug-induced tardive dyskinesia (25), potassium channel mutations and drug-induced dysrhythmias (26), and polymorphism in the ryanodine receptor and anesthesia-induced malignant hyperthermia (27). Polymorphisms in genes of pathogenic agents (human immunodeficiency virus, bacteria, tuberculosis, and others) are another important source of genetic variation in drug sensitivity, but this review focuses only on polymorphisms in human genes that determine an individual's response to specific medications.

Table I provides examples of genetic polymorphisms in drug targets that have been linked to altered drug sensitivity. It is anticipated that ongoing studies will rapidly expand the number of such pharmacogenomic

relations. Furthermore, these examples represent monogenic determinants of drug effects, which are the easiest to recognize in population studies. It is likely, however, that drug response is often a polygenic trait, in which case more comprehensive studies will be required to define pharmacogenomic traits that are determined by multiple polymorphic genes. It should also be recognized that not all studies have reached the same conclusions about the effects of genetic polymorphisms on drug response [for example, not all studies of ACE polymorphisms have found a relation with response to ACE inhibitors (18)J. Such discordant results may be due to a number of factors, including the use of different end points in assessing response, the heterogeneous nature of diseases studied, and the polygenic nature of many drug effects. The rapidly expanding knowledge of the human genome, coupled with automated methods for detecting gene polymorphisms, provides the tools needed to elucidate these polygenic determinants of drug effects, thus fueling the burgeoning field of pharmacogenomics.

Relevance to Drug Discovery and Clinical Therapeutics

Substantial investments are being made within the pharmaceutical and biotechnology industries to use genomic strategies for the discovery of novel therapeutic targets (28). It is anticipated that, over the next decade, the Human Genome Project, coupled with DNA array technology, high-throughput screening systems, and advanced bioinformatics, will permit rapid elucidation of complex genetic components of human health and disease. Common polymorphisms in drug targets dictate that DNA sequence variations be taken into account in the genomic screening processes aimed at new drug development. This will provide new insights for the development of medications that target critical pathways in disease pathogenesis and medications that can be used to prevent diseases in individuals who are genetically predisposed to them.

Such pharmacogenomic studies should also permit the development of therapeutic agents targeted for specific, but genetically identifiable, subgroups of the population. This represents a migration from the traditional strategy of trying to develop medications that are safe and effective for every member of the population, a strategy that aims to provide a marketing bonanza but one that is a pharmacological long shot because of highly potent medications, genetically diverse patients, and diseases that have heterogeneous subtypes. Although debate about the wisdom of developing medications for only a subset of the population remains within the pharmaceutical industry (28), it is clear that science and technology will soon make it feasible to use molecular diagnostics to more precisely select medications and dosages that are optimal for individual patients (29). In this regard, automated systems are being developed to determine an individual's genotype for polymorphic genes that are known to be involved in the pathogenesis of their disease, in the metabolism and disposition of medications, and in the targets of drug therapy. Such diagnostics, which need be performed only once for each battery of genes tested, can then become the blueprint for individualizing drug therapy. This is illustrated in Fig. 3, which depicts various genes that could be genotyped to guide the selection and dosing of chemotherapy for a patient with acute lymphoblastic leukemia (ALL). It is already known that genetic polymorphisms in drug-metabolizing enzymes can have a profound effect on toxicity and efficacy of medications used to treat ALL (6) and that individualizing drug dosages can improve clinical outcome (30). It has also been established that the genotype of leukemic lymphoblasts is an important prognostic variable that can be used to guide the intensity of treatment (31). Furthermore, genetic polymorphisms are also known to exist for cytokines and other determinants of host susceptibility to pathogens, and polymorphisms in cardiovascular, endocrine, and other receptors may be important determinants of an individual's susceptibility to drug toxicity. Putting all of these molecular diagnostics on an "ALL chip" would provide the basis for rapidly and objectively selecting therapy for each patient. These examples represent our current, relatively poor, understanding, of genetic determinants of leukemia therapy and host sensitivity to treatment; ongoing studies will provide important insights that should substantially enhance the utility of such pharmacogenomic strategies for ALL and many other human illnesses.

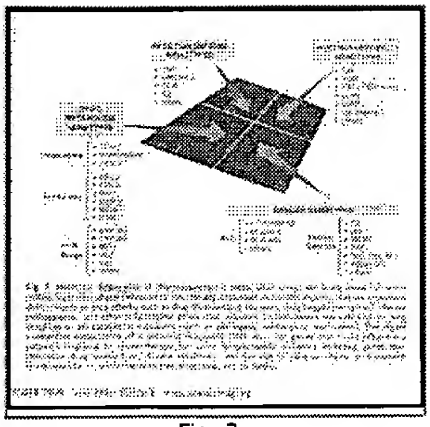

Fig. 3.

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[Author note]

William E. Evans* and Mary V. Relling

[Author note]

Department of Pharmaceutical Sciences, St. Jade Children's Research Hospital, Memphis, TN 38105, USA, and Colleges of Pharmacy and Medicine, University of Tennessee, Memphis, TN 38105, USA.
*To whom correspondence should be addressed. Email: william.evans@stjude.org

Conventional and genetic laboratory tests used to guide antimicrobial therapy

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[Headnote]

Detection of antimicrobial resistance is important so that clinicians can make rational decisions about optimal antimicrobial therapy for their patients. During the past decade, new types of antimicrobial resistance have emerged, some of which present new challenges for the clinical microbiology laboratory. In most cases, conventional culture-based testing methods continue to be useful. In other situations in which the organism responsible for infection grows slowly (for example, Mycobacterium tuberculosis), culture methods are technically difficult (such as for human immunodeficiency virus), or genotypes are inconsistently expressed (for instance, methicillin resistance in staphylococci), genetic susceptibility testing methods may offer special advantages. Determining serum concentra

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tions of antimicrobial agents may be useful both to ensure adequacy of treatment and to prevent toxicity. In this review, methods are described for conventional and genetic tests used to guide antimicrobial therapy.

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AIDS = acquired immunodeficiency syndrome; BHI = brainheart infusion; HIV = human immunodeficiency virus; MBCs = minimal bactericidal concentrations; MIC = minimal inhibitory concentration; MRSA = methicillin-resistant Staphylococcus aureus; NCCLS = National Committee for Clinical Laboratory Standards; PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism; SSCP = single-strand conformation polymorphism

During the past decade, new types of antimicrobial resistance have emerged. Vancomycin-resistant enterococci were first identified in Europe in 1987 and now account for a substantial percentage of enterococcal infections in many health-care facilities in the United States.' Methicillin-resistant Staphylococcus aureus (MRSA) infections continue to cause considerable morbidity and mortality, especially in institutionalized patients, and recently, MRSA strains with low-level resistance to vancomycin have been identified in the United States and Japan.23 A survey conducted at 30 US sites in 1996 showed that 46% of blood isolates of Streptococcus pneumoniae were either resistant or intermediately susceptible to penicillin.' Some Enterobacteriaceae-most notably, strains of Klebsiella species-have been demonstrated to possess extendedspectrum beta-lactamase resistance.4-6 Therefore, extendedspectrum beta-lactams such as ceftazidime may not be useful for treating infections caused by these organisms. Helicobacter pylori infection is now recognized as one of the most common chronic infections in humans. This pathogen has been shown to be increasingly resistant to clarithromycin and metronidazole.7'8 Appreciable frequencies of resistance to clindamycin, penicillins, cephalosporins, and cephamycins have been documented for strictly anaerobic bacteria, such as Bacteroides fragilis.9

Resistance among other organism groups has also been a challenge. Multidrug resistant strains of Mycobacterium tuberculosis emerged in the eastern United States in the early 1990s. Some of these strains were shown to be resistant to all frontline drugs, including isoniazid, rifampin, streptomycin, ethambutol, and pyrazinamide.10,11 Resistance of the herpesviruses to acyclovir12 and ganciclovir'3 is of particular concern for immunocompromised patients, including those coinfected with human immunodeficiency virus (HIV). Imidazole resistance among Candida species has also been documented.l4-6 Finally, the emergence of resistance to anti-HIV drugs, including reverse transcriptase and protease inhibitors, occurred almost as quickly as these drugs became available.17,18

Detection of these various types of antimicrobial resistance has become increasingly difficult for the clinical microbiology laboratory. In most cases, conventional susceptibility testing methods continue to be

useful. In other instances, however, especially those in which the organism grows slowly (for example, M. tuberculosis), culture methods are technically difficult (such as HIV), or genotypes are not always expressed (for example, MRSA), genetic testing methods may offer special advantages.

There are two major objectives of this report. The first is to present the principles of conventional culture-based susceptibility testing methods. These methods, except for the epsilometer test, have been used for many years, and general readers are assumed to have some familiarity with them; therefore, an in-depth critical discussion is not presented. The second objective is to discuss both the principles and the potential usefulness of recently developed genetic-based susceptibility testing methods. It is assumed that most readers have little knowledge in this area; thus, the discussion of this material is generally more developed than that for conventional methods. Although few genetic susceptibility testing methods are currently in use and available tests are performed mostly by reference laboratories, it is anticipated that these tests will soon become commonplace in many laboratories throughout the United States.

CONVENTIONAL (PHENOTYPIC) SUSCEPTIBILITY TESTING METHODS

Most conventional susceptibility testing methods assess the in vitro effects of antimicrobial agents on the growth of microorganisms or directly determine the presence of antimicrobial modifying enzymes. In many cases, these tests assess antimicrobial resistance phenotypes. Therefore, they reflect the expression of antimicrobial resistance encoded by genetic material that may be intrinsic or acquired.

The National Committee for Clinical Laboratory Standards (NCCLS) provides guidelines for preparation of media, incubation factors, and interpretation of results for the most commonly used susceptibility test methods-disk diffusion, broth dilution, and agar dilution.[9-23] Currently, approved standards are available and updated periodically for selected aerobic and anaerobic bacteria and yeasts. Tentative NCCLS guidelines exist for mycobacteria.[24] Mueller-Hinton medium is the most frequently recommended basal culture medium for testing bacteria that grow aerobically with these methods. Specialized media are required for Haemophilus species (Haemophilus testing media), S. pneumoniae (cation-adjusted Mueller-Hinton medium with lysed sheep blood), screening of Enterococcus species for vancomycin resistance (brain-heart infusion [BHI] medium), Neisseria gonorrhoeae (GC agar base and 1% defined growth supplement), mycobacteria (Middlebrook 7H 10 medium), and yeasts (RPMI medium). Currently, NCCLS guidelines are not available for gradient diffusion testing (epsilometer testing) or genetic testing methods for any microorganisms.

Disk Diffusion

In the classic technique of disk diffusion, the antimicrobial agent diffuses from a cellulose filter paper disk onto a solid medium surface over which bacteria have been streaked (Fig. 1). After 18 to 24 hours of incubation, inhibition of bacterial growth around the disk, referred to as the "zone of inhibition," is measured. This test method provides a qualitative result only-that is, on the basis of the zone of inhibition, the organism tested can be considered resistant, susceptible, or intermediately susceptible to the antimicrobial agent. In contrast, dilution test methods provide quantitative results-that is, a minimal concentration of the tested antimicrobial agent that inhibits growth of the organism (minimal inhibitory concentration [MIC]) can be determined (see subsequent discussion).

Broth Dilution

The broth dilution method can be performed manually by using standard-size test tubes (macrobroth dilution) or manually or automatically by using microtiter plates (microbroth dilution). In the macroscopic method (Fig. 2), various concentrations of antimicrobial agents are added to test tubes containing liquid

medium to which a standard inoculum of the test microorganism is also added. For most bacteria that grow aerobically, the test tubes are incubated for 18 to 24 hours. Longer incubation periods are required for anaerobic bacteria, mycobacteria, and yeasts. The lowest concentration of antimicrobial agent at which no visible growth is noted is referred to as the MIC.

In the microbroth formats, the same principles are followed as for the macrobroth dilution method, but assessments for growth are made by direct visualization of microtiter wells or by photographic density analyses. Automated or semiautomated methods based on the principle of microbroth dilution have been developed. These methods combine short incubations with automated reading of results. The Vitek System (bioMerieux Vitek, Hazelwood, Missouri) and the Baxter Microscan (Baxter Diagnostics Inc., West Sacramento, California) are examples of two of these methods that are commonly used in North America.

A specialized radiometric growth indicator method (BACTEC, Becton Dickinson, Sparks, Maryland) has been used for mycobacteria. For this method, the metabolism of 14C-labeled palmitic acid by mycobacteria in the presence and absence of inhibitory antimicrobial agents is measured. Mycobacteria metabolize palmitic acid and produce '4C02, which is measured by this system. Recently, another brothbased system has been developed for determining the susceptibility of mycobacteria to antimicrobial agents.25 This method uses a fluorescent indicator to assess the growth of mycobacteria in the presence of an antimicrobial agent and is referred to as the Mycobacterium growth indicator tube method (Becton Dickinson).

Agar Dilution

Like the broth dilution method, agar dilution provides a specific MIC; however, antimicrobial agents are incorporated into a solid (agar-containing) medium rather than dispersed in a liquid medium (Fig. 3). A standard concentration of organisms is "spot" inoculated onto the surface of this medium contained in Petri dishes. For most bacteria that grow aerobically, the agar plates are incubated 18 to 24 hours and then examined for growth. For anaerobic bacteria and mycobacteria (macrobroth dilution is the preferred method for yeasts), longer incubation periods are required. No growth of the test organism indicates that it is susceptible at the antimicrobial concentration incorporated into the medium.

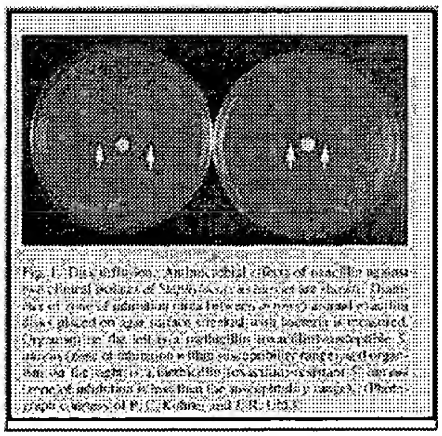

Fig 1

Semiautomated formats have been developed for the agar dilution method, which allows for large-scale batch testing. If the test organism is a slowly growing Mycobacterium, a modification of the agar dilution method referred to as the proportional method is used. An agar plate is subdivided into quadrants. Various quadrants contain different concentrations of the antimicrobial agent to be tested, and one

quadrant is free of an antimicrobial agent. The percent resistance of the mycobacteria tested against a specific concentration of antimicrobial agent is calculated by dividing the number of colonies on the antimicrobialcontaining quadrants by the number of colonies growing on the control quadrant and multiplying the answer by 100. The lowest concentration (pg/mL) of antimicrobial agent to which the test organism exhibits less than 1% resistance is referred to as the MIC.

Gradient Diffusion (Epsilometer Testing)

For this recently developed susceptibility testing method (commercially available as the E test, AB Biodisk NA, Piscataway, New Jersey), a gradient of increasing concentrations of the test antibiotic is incorporated into a single plastic-coated strip (Fig. 4). The strip is placed on solid agar onto which the test organism has been streaked. Experience with this method has been limited to aerobic and anaerobic bacteria. After 18 to 48 hours, the MIC is determined by visually identifying the intersection of the lowest point of the elliptical zone of growth inhibition and the gradient strip. A larger range of antimicrobial concentrations can be tested with this method than is practical by the broth or agar dilution methods.

Assessment of beta-Lactamase Activity

Direct detection of beta-lactamase activity for a specific microorganism may be of use to clinicians, particularly when serious life-threatening infections are encountered. The most commonly used assay method is the Cefinase chromogenic disk method (Becton Dickinson Microbiology Systems, Cockeysville, Maryland). This method relies on visualization of a colored product that results from hydrolysis of the substrate 3-lactam molecule, nitrocefin, contained in a paper disk. A loopful of organisms is smeared onto the disk, and if beta-lactamase is present, the nitrocefin is hydrolyzed and produces a color change. This method is reliable for detecting beta-lactamase-mediated resistance to penicillinase-labile penicillins, including penicillin, ampicillin, amoxicillin, azlocillin, carbenicillin, mezlocillin, piperacillin, and ticarcillin. Organisms that are frequently tested include Bacteroides species, Enterococcus species, Haemophilus influenzae, Moraxella catarrhalis, N. gonorrhoeae, and Staphylococcus species. Combinations of beta-lactams and inhibitors of beta-lactamases (sulbactam or clavulanic acid) may be useful for treating these organisms if they contain beta-lactamases. Standard methods, as previously discussed, should be used to test these antimicrobial combinations against these bacteria.

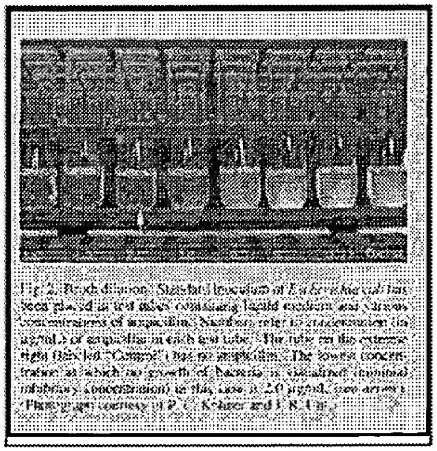

Fig. 2.

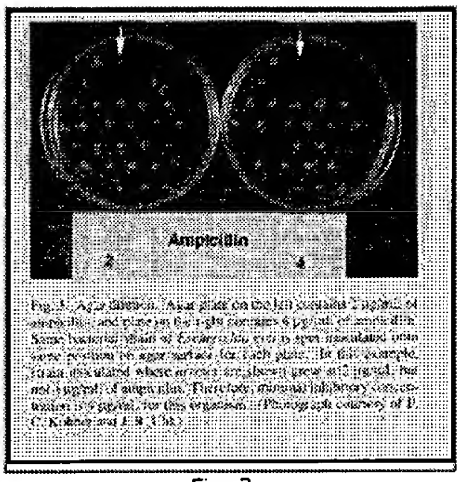

Fig. 3.

OTHER PHENOTYPIC SUSCEPTIBILITY TESTING METHODS

Tests for Antimicrobial Synergy

Antimicrobial synergy occurs when the effect of the combination of different antimicrobial agents exceeds the individual effects of the two agents added together. A commonly used synergistic combination of antimicrobial agents, trimethoprim and sulfamethoxazole, has been commercially available for many years. For this drug combination, conventional disk diffusion or dilution (broth or agar) testing methods can be used, inasmuch as appropriate ratios of the two drugs for testing by these methods have been standardized. 19

The combination of a penicillin, ampicillin, or vancomycin with an aminoglycoside for the treatment of serious enterococcal infections, such as endocarditis, is well established. Some enterococcal strains have high-level resistance to aminoglycosides; therefore, combination with a penicillin produces no synergistic effect. If this high-level aminoglycoside resistance is detected, then more complete synergy studies (see subsequent discussion) are unnecessary. High-level aminoglycoside resistance is defined by the NCCLS"9 as growth of organisms up to 6 mm within disks containing 120 jg of gentamicin or 300 jg of streptomycin placed on Mueller-Hinton agar or growth of organisms in BHI broth or on BHI agar each containing 500 Rg/ mL of gentamicin or in BHI broth or on BHI agar containing 1,000 gg/mL or 2,000 Rg/mL of streptomycin, respectively. The NCCLS guidelines also indicate that other aminoglycosides need not be tested because their activities are not superior to gentamicin and streptomycin.'9

Refined methods for testing other antimicrobial combinations against bacteria that grow aerobically (including checkerboard and time-kill studies) are not currently provided by the NCCLS. A detailed discussion of these methods is beyond the scope of the current article, and interested readers are referred to a comprehensive review provided by Eliopoulos and Moellering.26

Tests for Minimal Bactericidal Activity

Minimal bactericidal concentrations (MBCs) are determined by subculturing all tubes in a broth dilution-antimicrobial agent susceptibility test series in which growth of bacteria has been inhibited, including the tube with the MIC and all tubes with greater concentrations of antimicrobial agent. The subcultures are transferred to agar plates free of antimicrobial agents, which are then incubated to determine whether viable organisms were present in the original tube. The first tube or the lowest concentration that yields no growth of organisms (or reduction of the initial inoculum size by 99.9%) on

the subculture plates is considered the MBC. If the MIC and the MBC are identical or almost identical, the antimicrobial agent is considered bactericidal for that particular organism. If the MBC is substantially greater than the MIC, the drug is considered bacteriostatic.

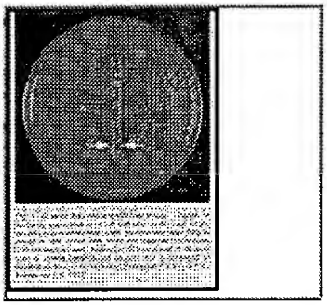

Fig. 4.

The validity of determining the bactericidal activity of an antimicrobial agent remains controversial. In fact, the accepted definition for MBC-that is, the reduction by the test antimicrobial agent of an initial bacterial inoculum by at least 99.9%is arbitrary. Nevertheless, some studies suggest that the MBC determination may be useful for serious or difficult-to-treat infections, such as endocarditis, meningitis, or osteomyelitis.27 Although the NCCLS provides tentative guidelines for this test method,2' reproduction of results with use of these standards may be difficult.

In general, clinicians can rely on information about bactericidal or bacteriostatic activity obtained during research and development of new antimicrobial agents. Some physicians, however, request that the clinical laboratory determine the MBC for individual clinical isolates; this MBC is then used as a predictor of efficacy of therapy. In such circumstances, the MBC is usually related to assayed serum levels of the antimicrobial agent being administered (see subsequent discussion). In the past, such efforts have been hampered by the lack of a standardized method for determining MBCs and by the absence of data that substantiate a good correlation between MBC results and clinical outcomes.

The problem has been complicated by reports of the phenomenon of antimicrobial "tolerance."28 Tolerance, which has been noted with staphylococci and streptococci, is said to be present when an antimicrobial agent that is usually bactericidal fails to kill a particular organism at concentrations that approach the MIC. One definition of tolerance is an MBC-to-MIC ratio of 32 or more.28 Some physicians are concerned that lack of an optimal response to therapy in a specific patient may be due to the presence of tolerance, and the laboratory is asked to determine MBCs to investigate this possibility.

Our experience at the Mayo Clinic suggests that failure of antistaphylococcal therapy is rarely caused by tolerance. Some support for the likelihood that treatment will be effective can be obtained by performing assays (see subsequent discussion) to demonstrate the presence of adequate serum antimicrobial levels.

Serum Bactericidal Test

In the serum bactericidal test, samples (peak and trough) of the treated patient's serum are incubated (in doubling dilutions with broth or serum) with an inoculum of the infecting organism to determine the highest dilution (titer) that is bactericidal. Serum specimens can be obtained to coincide with anticipated peak and trough antimicrobial levels. This test is, in effect, an assay of the activity of the drug in the serum against the patient's infecting microorganism. Indirectly, it assesses the combined factors of the susceptibility of the test organism and the serum concentration of the antimicrobial agent as well as the interaction between serum and organism and serum and antimicrobial agent. Like the minimal bactericidal test, the serum bactericidal test has prompted persistent questions about clinical utility; however, tentative

NCCLS guidelines now exist for this method.22

The serum bactericidal test has been proposed as a means of monitoring the bactericidal antimicrobial therapy that is thought to be important in the treatment of bacterial endocarditis, infections in immunocompromised patients, meningitis, and osteomyelitis.28 Furthermore, the serum bactericidal test is considered useful for measuring the combined effect of several antimicrobial agents being administered concurrently. Additional studies (including correlations of specific titers with outcomes) are needed before the true value of this test can be determined in these various situations.

In the past, the serum bactericidal test was used most often as a guide to treatment of bacterial endocarditis, but investigators disagreed about which titers (dilutions of the serum) were indicative of significant bactericidal activity. In one study, the frequently mentioned value of 1:8 had no significant association with outcome, whereas peak serum bactericidal titers of 1:64 or more corresponded with complete bacteriologic cure.29 In that same study, however, the investigators indicated that the serum bactericidal test cannot predict either bacteriologic failure or clinical outcome. Until clinical studies have substantiated the value of the proposed standardized method, it may be most prudent for diagnostic laboratories to refrain from performing serum bactericidal tests. Assays of serum concentrations of antimicrobial agents and perhaps the MBC probably are the currently available tests that, in conjunction with the patient's clinical course, can provide some support for the adequacy of therapy in bacterial endocarditis.

GENOTYPIC SUSCEPTIBILITY TESTING METHODS

Recently, genetic susceptibility test methods have become available. For several reasons, these methods have the potential to provide a more rapid and reliable assessment of antimicrobial resistance in comparison with phenotypic testing methods. First, genetic tests can be performed directly from clinical specimens, and isolation of an organism by culture becomes unnecessary. Second, genetic methods assess the "genotype" of the organism, whereas conventional susceptibility techniques assess the "phenotype" or expression of the genotype under artificial or laboratory conditions. Although debate exists among authorities about which of these assessments is more clinically relevant, it seems reasonable that the lowest-risk approach for the patient is to determine the genotype. This strategy may be especially important if one is dealing with serious life-threatening infections such as meningitis or bacteremia or infections necessitating prolonged courses of antimicrobial therapy such as endocarditis or osteomyelitis. Third, in some cases, genotypes may be discerned long before phenotypes can be determined because of the slow growth of an organism. Fourth, some organisms are not easily cultured or cannot be cultured; therefore, only genotypes can be determined. Finally, genetic methods may lessen the biohazard risk that may occur with the propagation by culture of an organism that is required for conventional test methods.

Specific examples for which genetic methods have been demonstrated to be more accurate than conventional phenotypic methods include the assessment of methicillin resistance in coagulase-negative Staphylococcus species,3" low-level vancomycin resistance in Enterococcus species,31 or extended-spectrum (-lactamase resistance in gram-negative facultatively anaerobic bacteria such as Escherichia coli and K. pneumoniae.45 Because of the slow growth of M. tuberculosis, conventional susceptibility testing for isoniazid, rifampin, ethambutol, streptomycin, pyrazinamide, and the fluoroquinolones requires weeks to months to complete. In contrast, the assessment of mutations in genes associated with resistance to these drugs can be performed directly from a clinical sample in as little as 1 working day.32^4 Another advantage of detection of drug resistance in M. tuberculosis by this means is that it lessens the biohazard risk in comparison with conventional methods using cultures. Because viral replication (culture) assays are technically difficult, the detection of mutations in genes associated with resistance to antiviral drugs is a convenient means by which resistance can be rapidly and accurately

determined. Examples include the assessment of mutations in either the thymidine kinase or the DNA polymerase genes of herpes simplex virus, which are associated with acyclovir resistance;l2,47 within a protein kinase genetic locus (UL97) in cytomegalovirus, which are associated with ganciclovir resistance;'348 or within the reverse transcriptase and protease genes of HIV, which are associated with resistance to reverse transcriptase and protease inhibitors, respectively.49 Although a direct mechanistic link between nucleotide sequences in hepatitis C genomes and effect of immunotherapy (interferon) has not been proved, such an association seems to predict response to therapy.5o Hepatitis C is a nonculturable pathogen; hence, this association may represent an application of genetic testing that is not possible with use of conventional culturebased susceptibility testing methods.

In some instances, genetic testing methods may have less utility than conventional susceptibility test methods. (1) They may lack sensitivity when few organisms are present in a sample. (2) Different assays are required for each antimicrobial agent tested (individual antimicrobial agents may also be associated with multiple target genes or a large array of mutations). (3) A genetic method for resistance for some antimicrobial agents may not have been defined. (4) False-positive results may occur because of contamination of the sample with extraneous nucleic acid. This last-mentioned problem is of particular concern when nucleic acid amplification techniques such as polymerase chain reaction (PCR) are used. The specificity, however, has been considerably enhanced with the development of enzymatic and chemical sterilization techniques for amplified nucleic acid.51 The development of methods for concentrating nucleic acids from large volumes of clinical specimens should address the first point about sensitivity.

Most genotypic methods include a step in which the "target" nucleic acid is amplified. This process is usually accomplished with use of PCR. The product of this reaction, referred to as an amplicon, can then be confirmed as the desired target DNA (that is, part or all of a resistanceassociated gene) by electrophoretic mobility determinations, probe hybridization assays (Southern blotting, slot, dot-blot, enzyme-linked immunosorbent assay, or liquid hybridization formats), restriction fragment length polymorphism (RFLP) analysis, or DNA sequencing formats. The following discussion describes the more common genetic methods reported for detecting antimicrobial resistance. Some of these methods have already been introduced into the clinical microbiology laboratory. The addition of others will depend on how they compare with conventional methods in performance characteristics (accuracy), turnaround time for results, and cost.

Detection of Antimicrobial Resistance Genes by PCR Amplification of Target DNA and Amplicon Confirmation by Gel Electrophoresis, Probe Hybridization Techniques, or DNA Sequencing

An example of a multiplex PCR method that we have used to detect the mecA gene in staphylococci, which have been isolated with use of culture methods, is shown in Figure 5,30.32 The mecA gene encodes a penicillin-binding protein, designated 2a or 2', which has a decreased affinity for methicillin or related compounds (nafcillin, oxacillin, cloxacillin, and dicloxacillin). Two distinct PCR amplifications are produced simultaneously in the same reaction tube-hence the term "multiplex PCR." In one PCR, a portion of the mecA gene is amplified, and in the other PCR, a nucleic acid sequence of the 16S ribosomal RNA gene unique to staphylococci is amplified. The PCR amplicons are electrophoresed through an agarose gel and stained with ethidium bromide; then sizes are determined by comparison with standard DNA fragments. A "positive" result should produce amplicons of mecA and 16S ribosomal DNA nucleic acid sequences of the sizes expected based on oligonucleotide primers used for the PCR. Additional confirmatory steps not shown in Figure 5 can be performed. The mecA amplicon can be subjected to probe hybridization or RFLP analyses, or its DNA sequence can be determined. In our experience, however, these steps have not been shown to add sensitivity or specificity to the assay.

Probe hybridization involves the use of labeled segments of nucleic acids that hybridize to the target DNA (nucleic acid probes). This process can be accomplished by classic Southern blotting of electrophoretic gels or by solid-phase (slot, dot-blot) or solution-phase hybridization formats. For the last two methods, gel electrophoresis of the amplicons is not required. For all of these methods, probes can be radiolabeled, nonradiolabeled, enzyme-coupled, or chemiluminescent. Readers are referred elsewhere for an in-depth discussion of these methods.53 54 Amplified DNA can be confirmed as the appropriate target nucleic acid by DNA sequencing (see subsequent discussion).

Detection of Antimicrobial Resistance Genes by the Branched DNA Method

The branched DNA method (a non-PCR-based method) is commercially available (Chiron Diagnostics, East Wampole, Massachusetts) for the determination of blood levels (viral load) of HIV and hepatitis C virus. Recently, we have found this assay to be useful for detecting the mecA gene directly from blood cultures containing Staphylococcus species.55 This assay relies on the amplification of a signal and not target nucleic acid (Fig. 6). Signal generation is proportional to the amount of mecA gene sequences in the sample, which in turn is proportional to the number of staphylococcal cells used in the assay.

Detection of Antimicrobial Resistance Genes by PCR-RFLP Analysis

In RFLP analysis, the amplified DNA is digested (fragmented) with use of restriction enzymes (restriction endonucleases). These enzymes will cleave DNA molecules only at specific sites-that is, unique, short segments of nucleic acid. Therefore, if the nucleic acid sequence of the target DNA is known, RFLP analysis can be used to confirm the target DNA in its amplified form. We have used RFLP analysis to distinguish among genes that encode vancomycin resistance.56 Because of conserved DNA sequences that exist among vancomycin resistance genes, more than one vancomycin resistance gene may be amplified by using the same PCR oligonucleotide primers. RFLP analysis then can be used to distinguish the vancomycin resistance gene amplicons, providing that specific cleavage sites for a restriction endonuclease may exist in one but not another vancomycin resistance gene. Restriction fragments produced with use of the restriction enzyme MspI for vancomycin resistance genes vanA, vanB, vanC-I, and vanC-213 are shown in Figure 7 Upper, and the electrophoretic gel RFLP patterns obtained with MspI and various control strains and clinical isolates of Enterococcus species containing vancomycin resistance genes are shown in Figure 7 Lower. A potential advantage of the RFLP technique is that PCR amplicons may be sterilized-if digestion of the amplicon by a restriction enzyme occurs to the extent that annealing sites are no longer available for oligonucleotide primers used in subsequent PCRs.

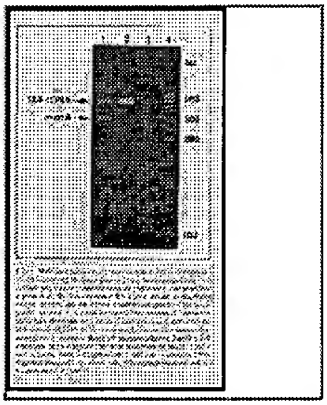

Fig. 5.

Detection of Antimicrobial Resistance-Associated Mutations by PCR-RFLP

Amplicons produced by PCRs can also be assessed for specific mutations associated with antimicrobial resistance by RFLP analysis. As an example, we and others have shown that point mutations in the catalase-peroxidase (katG) gene of M. tuberculosis are associated with isoniazid resistance.32-39 Two particular missense mutations occur with considerable frequency and can be identified by using the restriction endonuclease MspI.34-3s These mutations are located in codons 315 (serine--threonine) and 463 (arginine-leucine). In each of these cases, different restriction fragments occur in comparison with the wild-type allele (Fig. 8). In other words, specific DNA sequence interrogation at this site is possible by virtue of RFLP analysis.

Detection of Antimicrobial Resistance-Associated Mutations by PCR-Single-Strand Conformation Polymorphism

PCR-single-strand conformation polymorphism (SSCP) was first used to detect single nucleotide substitutions in human genes associated with hereditary disorders such as cystic fibrosis58 and phenylketonuria.5960 Recently, this method has been used to screen for point mutations in the M. tuberculosis genome associated with resistance to isoniazid (katG),3233 rifampin (rpoB),4 ethambutol,46 and the quinolones (gyrA).45 We recently compared this method with PCR-RFLP for the detection of the 463 codon mutation (arginine-leucine) in katG of M. tuberculosis and found the technique to be 100% sensitive and specific."

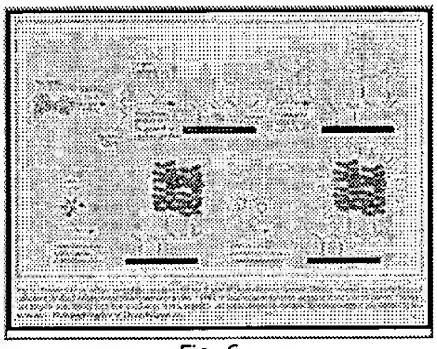

Fig. 6.

With this method, mutations are detected by the mobility shift of single-stranded DNA in nondenaturing polyacrylamide gel electrophoresis. After amplification of the target sequence by PCR, it is denatured to a single strand before being subjected to electrophoresis. Mutations are inferred by the appearance of bands at positions that differ from those observed with the wild-type strain (Fig. 9). We have also determined that the size of the PCR product is a critical factor for resolving differences of the denatured single-stranded DNA. In our experience, the optimal PCR product is in the range of 310 to 320 base pairs.61 The PCRSSCP technique can be automated by detection of fluorescein-labeled PCR products with automated DNA sequencing instruments.62

Detection of Antimicrobial Resistance-Associated Mutations by Universal Heteroduplex Generator Analysis

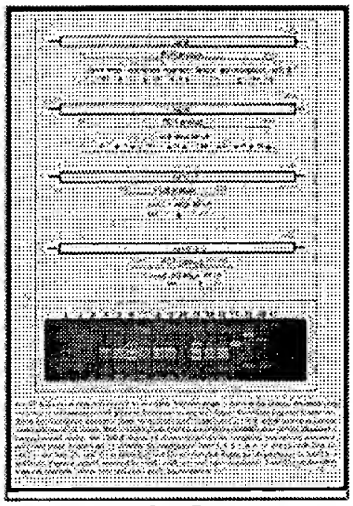

Fig. 7

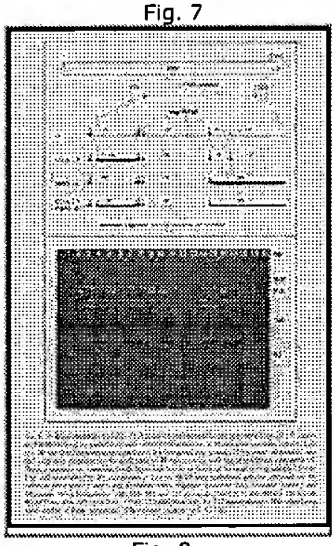

Fig. 8.

Universal heteroduplex generator analysis has been used to detect mutations in rpoB in M. tuberculosis.63 DNA from a universal heteroduplex generator and the test strain is denatured simultaneously in the same reaction mixture and then allowed to reanneal. The heteroduplex generator in the hypothetical example shown in Figure 10 Left has a 4-base pair deletion. When separate strands of DNA from the heteroduplex generator and the test strain reanneal, four separate hybridizations can occur. Two of these hybridizations (heteroduplexes) will result in double-stranded DNA, with a "bubble" occurring where no base-pair matches can hybridize. These hybrids will migrate as a single band when electrophoresed in a high-resolution gel. Two other bands will occur as a consequence of hybridization reactions (homoduplexes), which result in formation of the double-stranded DNA of the heteroduplex generator and the test strain. If mutations are present in the test strain, the positions of the homoduplexes may vary in comparison with homoduplexes formed when no mutations are present.

Detection of Antimicrobial Resistance-Associated Mutations or Genes by DNA Oligonucleotide Arrays on Silica Microchips

A promising new technology involves the synthesis of DNA probes directly onto silica microchips (Fig.11) 64 At present, more than 50,000 oligonucleotide probes can be synthesized directly onto these chips. In one method developed for HIV antiviral-associated resistance mutations, RNA is first reverse transcribed to complementary DNA, which is subsequently transcribed into RNA that is also labeled. These labeled RNA molecules are then hybridized to complementary probes, which have been synthesized

directly onto silica microchips. Where hybridization occurs, light is emitted. Such matrix hybridization formats have considerable potential for rapid comprehensive detection of resistance genes or mutations associated with antimicrobial resistance. This potential is especially notable if antimicrobial resistance in a specific organism can be due to multiple genes or multiple mutations (or both factors). Examples of this include isoniazid resistance for M. tuberculosis or resistance to reverse transcriptase or protease inhibitors for HIV, as presented in Figure 11. This technology also has the potential for automation and should thus be easily adaptable to most clinical microbiology laboratories.

Detection of Antimicrobial Resistance-Associated Mutations or Genes by DNA Sequencing

DNA sequencing remains the standard against which all of the foregoing methods are compared. Recent improvements in the DNA sequencing procedure have substantially reduced the time and cost for this method. Instruments are now available for semiautomated processing and analyzing sequencing gels. Readers are referred elsewhere for a comprehensive review of the subject.5,66

ASSAY OF ANTIMICROBIAL CONCENTRATION IN SERUM

Precise measurements of serum concentrations of antimicrobial agents may be necessary both to ensure adequate treatment and to prevent toxicity. Most often, these tests are useful when the margin between therapeutic and toxic levels of an agent is narrow, such as with aminoglycosides or vancomycin. They are also useful in patients with renal failure, who may accumulate high levels of agents that are primarily excreted by the kidneys. Administration of very high doses of penicillins or the presence of renal failure (or both factors) may prompt the monitoring of serum antimicrobial levels to prevent the neurotoxicity associated with excessive accumulations.

The utility of serum assays in verifying the adequacy of antimicrobial therapy has been studied. Moore and associates67 reported that achieving an adequate peak serum aminoglycoside concentration was the most important factor in predicting the outcome in gram-negative pneumonias. The same group demonstrated that a high peak serum concentration relative to the MIC for the infecting organism was a major determinant of the clinical response of other gramnegative infections to aminoglycoside therapy.67 The potential value of monitoring serum antimicrobial levels in guiding treatment of bacterial endocarditis has previously been mentioned. Providing adequate treatment for patients with acquired immunodeficiency syndrome (AIDS) who have pneumonia due to Pneumocystis carinii has necessitated the frequent monitoring of serum specimens for levels of trimethoprim and sulfamethoxazole.

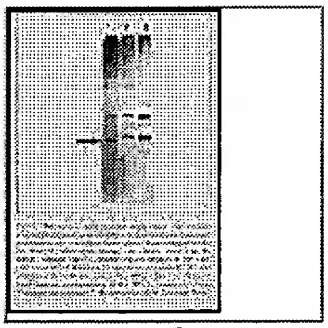

Fig. 9.

Serum assay values cannot be interpreted accurately without the provision of critical information by the clinical service. The dosage and route of administration of the antimicrobial agent (including the duration of intravenous infusion), the time of administration, and the time of collection of the specimen all must be documented. In addition, information about all drugs being administered must be provided. Trough levels

are usually obtained just before administration of the next dose of the agent. Peak levels may be expected 1 to 2 hours after oral administration of a dose, hour after intramuscular administration of a dose, and 30 minutes after the end of intravenous infusion of a dose. Clinicians must determine the value of obtaining peak or trough levels (or both). Both determinations may be indicated for aminoglycosides, and the peak may be most important for [-lactam antimicrobial agents. Routine requesting of both levels of all agents is not indicated and will result in the costly and inefficient generation of information that will have marginal utility.

Several suitable methods can be used to perform serum assays. Some techniques are most applicable to only certain agents. At the Mayo Clinic, a bioassay is used for metronidazole, erythromycin, and clindamycin. Bioassays are based on the comparison of a susceptible organism to the unknown concentration of the antimicrobial agent in the patient specimen with the response to a known antimicrobial concentration under identical conditions. Immunoassays are used for the aminoglycosides and vancomycin, and high-performance liquid chromatography is used for all penicillins and cephalosporins, 5-fluorocytosine, chloramphenicol, sulfonamides, trimethoprim, fluoroquinolones, itraconazole, and ganciclovir. Two commonly used immunoassays that are nonradioisotopic are the fluorescence polarization immunoassay and the enzyme-mediated immunoassay. In the former, a change in the nature of the activity of light on a substrate is measured. For the enzyme-mediated immunoassay, the alteration of activity of an enzyme is measured when an antimicrobial-enzyme conjugate is bound to an antibody to the antibiotic. The high-performance liquid chromatographic assay follows the principle of chromatography, whereby the drug in a gas phase is absorbed onto a liquid and quantitatively assessed.

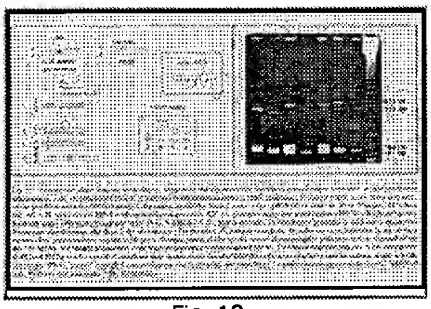

Fig. 10.

The choice of assay method will depend on the types and numbers of antimicrobial agents being tested in a specific laboratory, the technical expertise available, and the cost of equipment and reagents relative to the income for performing the tests. For example, a small laboratory that performs relatively few tests primarily for one or two aminoglycosides might use only bioassays or perhaps an immunoassay, whereas a large reference laboratory must be proficient in all available methods. Clinicians and laboratorians should be aware of and make note of all conditions that might influence assay results, including concurrent administration of other drugs and underlying illnesses that could interfere with assay methods. Full consideration of all elements involved in an assay, from proper timing of collection of a specimen to selection of the best method for the appropriate drug and the circumstances of the patient, will help ensure that an interpretable and useful result is obtained.

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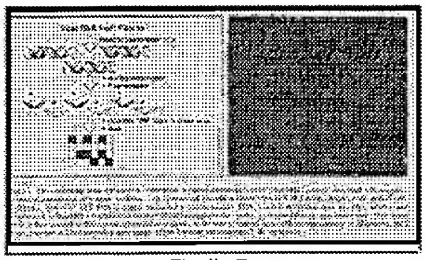

Fig.II. E

[Sidebar]

From the Division of Clinical Microbiology, Mayo Clinic Rochester, Rochester, Minnesota.
Individual reprints of this article are not available. The entire Symposium on Antimicrobial Agents will be available for purchase as a bound booklet from the Proceedings Editorial Office at a later date.

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The new biology: Histopathology

The Lancet; London; Jul 1999; Phil Quirke;Nic Mapstone;

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Future

Abstract:
Quirke and Mapstone discuss a new biology and its impact clinically on the histopathology subspecialties. The new biology will encompass a combination of novel genomic and proteomic data, advances in analysis, computation, bioinformatics, and new methods of microscopy and signal localization.

Full Text:

The new biology will encompass a combination of novel genomic and proteomic data, advances in analysis (eg, nucleic-acid arrays), computation, bioinformatics, and new methods of microscopy and signal localisation. The human genome may be available before the end of the year 2000 and will be rapidly followed by single nucleotide polymorphism sites (SNPS) that can identify points of variation between individuals. These data and the new techniques will have an impact on all areas of medicine.

How will these changes affect histopathology? Pathology, the study of disease, and the basis of clinical medicine and many of the fundamental processes underlying human disease, should yield to the tools of the new biology. Histopathology, the study of pathological processes in tissues, is well placed to benefit from such information. DNA and RNA can be analysed from haematoxylin and eosin sections, from fresh tissue or archival paraffin-embedded material, and from samples obtained at surgery, biopsy, cytopathology, or necropsy.1,2 DNA and RNA sequences can be localised in tissues by sensitive in-situ techniques, such as in-situ hybridisation and in-situ PCR amplification, and the tissue distribution of proteins can be simply and rapidly localised by immunocytochemistry. Many of the new techniques can probably be applied to archival material. Archival material has several advantages; not only is it from individuals for whom the disease process has been documented, but also data may be available about the outcome as well as the response to treatment and any associated diseases. Material available from well-controlled clinical trials would enable a wide range of hypotheses to be tested. Histopathology is a biological Klondike.

Before our colleagues decide to plan their early retirements, we must also remember that current molecular techniques reduce complex tissue phenotypes into simple stretches of DNA and RNA. Pathological processes are composed of many cell types and these may be in different stages of differentiation. Most molecular techniques "average out" the biological processes rather than identifying specific lesions in the cell lineage of interest. We must accept that in many situations the information from the new biology may not yield as much knowledge as that provided by expert morphological assessment. The new biology will only replace the microscope if the knowledge it yields is more valuable and more cost-effective.

The new biology will affect all areas of histopathology, including cytopathology and forensic pathology.

Histopathology

The new biology is starting to have an impact clinically on the histopathology subspecialities. The greatest effects have been in the areas of cancer, infection, and genetics.

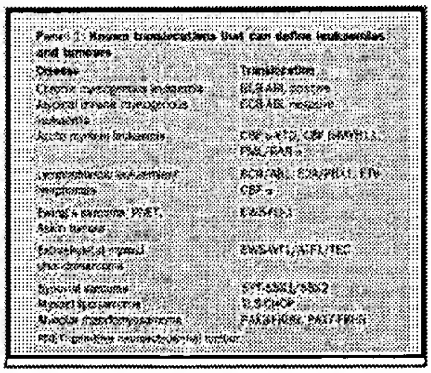
Cancer

Diagnosis and classification of tumours

The new biology is challenging our fundamental pathological concepts of early neoplasia. When does a reactive proliferation become a tumour? Is it the presence of a defined morphological pattern, the excess growth of a particular clone of cells (clonality), or a specific molecular lesion? Can an individual molecular lesion define a neoplasm?

The use of molecular techniques raises many difficulties in the early stages of neoplasia. We can identify abnormal clones of cells in morphologically normal mucosa as well as in metaplastic and dysplastic areas.3,4 Lesions at the cellular level can also be found in endometriosis,5 in normal breast tissue,6,7 and in non-neoplastic mucosa from patients with ulcerative colitis.8 Tissue from the latter group of patients can even show gross abnormalities of DNA content on flow cytometry9 before the appearance of dysplasia. Indeed both clonality and molecular lesions have been shown in endometriosis which is not deemed a neoplastic process.5,10

The finding of early genetic changes in histologically normal mucosa and non-neoplastic disorders should contain clinically useful information about the development of early neoplasia. Are genetic lesions alone enough to define the neoplastic state? A single mutation in an oncogene such as Kirsten ras can be found in simple non-neoplastic normal mucosa,11 colonic metaplastic polyps, and aberrant crypt foci12 as well as neoplastic adenomas and colorectal carcinomas. Inactivation of one copy of a tumour-suppressor gene occurs in all cells in patients with diseases such as hereditary retinoblastoma or familial adenomatous polyposis. Thus, one lesion does not appear to be enough. It takes a second hit to inactivate both copies of a tumour-suppressor gene and initiate carcinogenesis-but how many other lesions have to occur before there is a true neoplasm? Such questions cannot be answered yet but improved understanding of these processes will be provided by the new biology. We will probably have to revise our concepts of early neoplasia in the light of such data.



Panel 1:

Tumour classification needs substantial amendment in certain areas. Chromosomal and molecular definitions are already used to classify lymphomas, soft-tissue tumours, and paediatric tumours (panel 1)

and include such disparate entities as Ewing's sarcoma, Askin tumour, and primitive neuroectodermal tumours (PNET). For these tumours, the identification of a certain translocation defines the type of tumour and thus its therapy. This practice will probably become commonplace; current applications in soft-tissue pathology have been reviewed.13-15 However, these tumours are rare and are a small part of the workload of a district hospital.

What about common tumours? Interesting surprises are turning up even here. A new subtype of a common cancer with a different molecular mechanism and a different clinical behaviour has been described: the cancer due to a defect in mismatch repair.16 These tumours probably have a common underlying abnormality that leads to methylation of the promoter of hMLH-I, a key gene in the repair of small DNA mismatches.17,18 Methylation of the promoter stops production of the protein, induces a failure of mismatch repair, and expansions and contractions of repetitive sequences occur. This causes inactivation of a different pattern of tumour-suppressor genes to that found in the more common p53 pathway (panel 2). These tumours occur in the colon (15%), stomach (10-15%), ovary (3%), endometrium (9-22%), and at other sites.16 Tumours due to defects in mismatch repair may behave differently from those due to other mutations. In colorectal cancer, they have a different distribution (generally located in the right colon), a characteristic histological phenotype (mucinous or poorly differentiated), and an increased frequency of multiple, synchronous, and metachronous cancers, and they may have a better prognosis and respond differently to chemotherapy in vitro.19-21 The cause of the methylation in these tumours remains elusive. Knowledge of other molecular subtypes may improve understanding of the range of behaviour of these and other important cancers. Diagnosis of such mismatch repair abnormalities used to depend on molecular analysis for microsatellite instability but now rapid antibody methods are available.19 Different mechanisms of causation of cancer may require different approaches to treatment.22,23 Histopathologists will need to respond to such challenges.

Aetiology of cancer

Many cancers have a strong genetic influence but most arise through the interaction of chemical carcinogens and the DNA of stem cells. We already know the link between carcinogens and specific molecular defects24 such as aflatoxin and p53 mutations at codon 249, benzpyrene and p53 mutations at codons 157, 248, and 273, and ultraviolet-light injury and CC to TT thymidine-dimer formation. We face not only the prospect of diagnosing an individual's cancer but identifying the specific cause. However, knowing the cause is only the first step to prevention. Although the relation between squamous-cell carcinoma of the lung and smoking has been known for nearly 50 years, it still remains a challenge to reduce smoking in the population-even with overwhelming scientific evidence. Molecular epidemiology has been limited over the past 10 years by the obstacle that extensive DNA-sequencing studies represent. With the advances from the human genome project of large automated workstations for PCR, DNA-sequencing reactions, and higher throughputs on DNA sequencers, mutation analysis of populations will become much easier and expand our knowledge of this important area.

Many tumours arise because of either a weak or a strong genetic predisposition. We know some strong genetic predispositions to specific cancers and the molecular defect caused by inactivation of both copies of tumour-suppressor genes such as retinoblastoma (Rb), familial adenomatous polyposis (APC), neurofibromatosis (NF-1, NF-2), and Li Fraumeni (p53). This knowledge has become more important to histopathologists with the discovery of genes involved in commoner cancers such as BRCAI and BRCA2(25) in breast cancer, and h-MSH-2, hMLH-1, h-PMS-1, h-PMS-2, h-MSH6(26,27), the gene for the type II receptor of transforming growth factor beta,28 and polymorphisms in the APC gene29 in colorectal cancer.

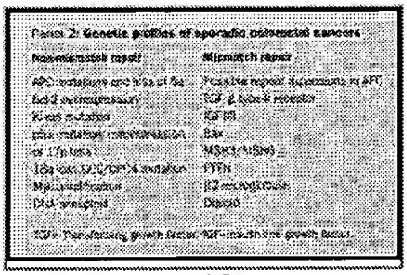
We are often asked whether a patient with a family history has a genetic predisposition. Our ability to

confirm a germ-line defect is currently limited but will be less so in the future. Simple tests may be developed that indicate which protein is abnormal and suggest methods for the rapid prediction of a certain gene in a patient-eg, the use of mismatch-repair antibodies to show loss of expression of the same mismatch-repair protein in tumours from multiple members of the family.

In the future, we will be faced with the identification of new genes or polymorphisms of known genes which have a smaller effect on the development of cancer. We may be asked to provide a diagnosis for such patients from tissue samples. We already know of one polymorphism that can disrupt APC function and predispose to colorectal cancer in Ashkenazi Jews.29 Polymorphisms will also be important in determining an individual's risk of cancer after exposure to a carcinogen such as N-acetyl transferase glutathione S-transferase,30 or response to therapy, or the possible toxicity of a particular treatment. The development of a single-nucleotide polymorphism map will identify the variable bases in the human genome that cause the above differences between individuals. Some 3X10^sup 5^ of these will be available in the next 2 years, with half of these positioned on the genome.31

Staging of tumours

Will the new biology help with the staging of tumours? Despite the promises of the molecular discoveries of the 1980s and 1990s, the staging of common cancers is still by dissection and histology. Molecular staging is a possibility since individual molecular lesions develop at different times in the progression of a tumour; however they do not always develop synchronously with standard histopathological staging and must be shown to yield more valuable clinical information. Individual lesions may provide little information but molecular profiles may be developed that offer additional value or superior information and will probably occur when we fully understand the processes of invasion and metastasis. Currently, we have only a patchy knowledge of their mechanisms.



Panel 2:

One area in which molecular techniques appear superior to standard methods is the prediction of minimum residual disease or early relapse by the identification of recurrent clonal populations in bone marrow or peripheral blood.32 There have also been suggestions that molecular analysis can predict spread of disease past the surgical margin in head and neck cancer" and that molecular lesions in peritoneal washings can identify patients with a high risk of relapse in gastric cancer.3" In the future, we may routinely take samples for molecular analysis not only from the surgical specimen and its margins but also, for molecular analysis, from other sites such as peripheral blood, or pelvic or peritoneal washings. However, a note of caution needs to be added. The presence of DNA alone in the peripheral blood or other bodily fluids will not always equate with the presence of cells capable of establishing viable metastases and mutations in oncogenes such as Kirsten ras are not always confined to malignant tumours and can be found in normal mucosa, metaplastic polyps of the colon, and adenocarcinomas.11,12 Experimental evidence also suggests that DNA can leach from tumours into regional lymph nodes and it may even get to the peripheral blood and give rise to false-positive molecular results.35 Careful large clinical studies in a range of tumour types with different targets are required to identify the true value of these assays.

Prediction of type of therapy and response

The histological assessment of oestrogen receptors by immunocytochemistry was rapidly taken up by histopathologists and we are now faced with identifying breast cancers with high amounts of amplification of c-erbB2 for antibody therapy.36,37 The new biology may bring other ways of predicting response to various chemotherapy regimens. This is particularly important as we move to more toxic and expensive treatments. A range of markers such as P glycoprotein38 has been claimed to predict response, but carefully conducted randomised studies on large numbers of patients are lacking. These markers need to have been compared with the current gold standard of high-quality histopathology and then reproduced elsewhere with different series of patients. Antibodies to the proteins of key genes in the repair of DNA damage and the apoptosis pathways (ie, mismatchrepair genes, p53, Bcl-2) as well as to key enzymes such as thymidylate synthase for fluorouracil, or topoisomerase 1 for irinotecan, need to be evaluated. Early studies of tumour-suppressor genes in patients treated for colorectal cancer suggest that deletions of 18q and of p53 induce resistance to chemotherapy.39 Cells in these patients may not be able to recognise DNA damage from chemotherapy due to failure of the apoptotic pathways. In the future, we may assess all tumours this way, with the molecular pathologist suggesting the type of therapy. If we do get good molecular treatments with gene-replacement therapy, we may need to identify the genetic lesions and susceptibilities of a tumour so that specific treatments can be undertaken. We may also be required to monitor the success of therapy by molecular techniques looking for the uptake of the therapeutic nucleic acid or by immunocytochemistry looking for products of reporter molecules in tumour biopsy samples.

Infection

Histopathology deals with tissues from many cases of infectious disease. We currently have valuable techniques for the detection of infectious agents in paraffinembedded tissue. We can amplify DNA for bacterial, viral, or non-culturable infectious agents40 from paraffinembedded tissue.2 Viral RNA can be reliably detected by the adoption of suitable methods such as glycogen carriage.41 We can, therefore, frequently identify and type an infectious agent definitively when the morphological changes suggest it. Currently this may take several hours, but newer thermal cyclers that use thin-film heaters to heat small volumes of solutions combined with TaqMan fluorescent dyes can shorten this to amplification and detection in 7 min and allow the use of quantitative PCR in the "field".42 This type of procedure is most valuable in acute infectious epidemics, or with agents such as tuberculosis in which current diagnosis by culture is extremely slow. These methods also allow us to identify non-culturable organisms such as Whipple's bacillus40 and expand our knowledge of its role in disease.

The new biology will not only identify currently unknown organisms but also allow us to obtain vast amounts of important information about the infectious organism and the host reaction to it. There are at least 11 bacterial genomes completely sequenced and 50 more projects underway. The complete genome of the tubercle bacillus is now known and such data should yield unprecedented information about the infective agent-eg, antibiotic sensitivity, risk of becoming dormant, ability to become multidrug resistant, and the source of infection. Information will also be provided by molecular assays of the host. Their predisposition and resistance to the organism may be accurately determined by their genotype, immune profile, and pattern of genetic response to the infection. For example, studies have suggested that patients who are heterozygotes for (delta)F508 are less susceptible to cholera,43 people with certain HLA types such as B27 may be more susceptible to intracellular infectious agents,44 HIV progression is affected by HLA type,45 and polymorphisms in interleukins or other cytokines may affect the efficiency of the immune response.46

Genetics

This will be dealt with elsewhere and will only be touched on briefly. Archival material can be used for genetic diagnosis and is frequently called on to check the genetic status of deceased relatives of index cases. Suspicious histological findings can be directly confirmed or excluded by molecular testing. For example, in cases of meconium ileus, the cystic-fibrosis mutation status can be established. New methods that simultaneously analyse multiple genes will allow genetic screening of histopathological material from a range of tissues, some of which are currently only cursorily investigated-eg, the diagnosis of the presence or absence of fetal parts in early miscarriages may be replaced by diagnostic tests that reveal the cause of the problem. Currently such techniques are confined to gross chromosomal lesions such as trisomies, but in the future it may be possible to do whole genome screens with expression arrays on such material to identify preventable causes of miscarriage.

The ability to investigate an individual's genetic predisposition to Huntington's disease, dementia, cardiovascular risk, or others, from any stored surgical or diagnostic material also opens up ethical problems for the histopathologist. We are custodians of an individual's genetic medical record. What rights do we have to dip into their DNA record to conduct scientific studies, how do we protect the rights of patients who do not know that we have such potential sources of information about them, who should have access to such material, and how do we ensure that access is ethical? Such questions are only now beginning to be raised.

Cytopathology

With advances in imaging and tissue sampling, we may need to do more cytopathological diagnosis and may need to stage and predict the response to therapy of tumours from ever smaller amounts of tissue. Indeed, we know that single cells may be used for genetic analysis at single47 or multiple sites,48 and for comparative genomic hybridisation.49 Repeated fine-needle sampling of metastatic deposits for chemosensitivity status may become routine feasible at both the nucleic acid and protein levels; the latter by immunocytochemistry where antibodies to proteins such as P glycoprotein are currently available. We do not yet know the sensitivities of nucleicacid arrays but even these may one day be available to use on single cells.

Screening

We are on the edge of a new era in cytopathology with automation of cervical cytology. The new biology may identify novel markers of neoplasia. Human papillomavirus (HPV) testing has already been suggested, since subtypes such as HPV 16 and 18 are associated with an increased risk of progression to cervical intraepithelial neoplasia. Specific high-risk genetic lesions linked to cervical intraepithelial neoplasia may also be found. HPV testing can be simply and rapidly done by ELISA, and other markers may be recognisable by automated systems such as Autopap. These currently screen only for abnormal morphological features but could readily be modified to look for immunocytochemical markers. Screening for colorectal cancer may also "go molecular" with multiplex molecular testing for genetic lesions in p53, APC, and Ki ras on exfoliated cells in stool samples. Saliva may also be used to test for oral cancers.50 Such methods have already been shown to be feasible with urine screening for bladder cancer51,52 and may rapidly replace urine and sputum cytology if the costs of the technology are reduced.

Forensic pathology

Forensic pathology has been at the forefront of molecular techniques in its use of DNA fingerprinting to

identify unique genetic patterns in nuclear and mitochondrial DNA. In the future, determination of the ethnic origin and possibly disease-susceptibility profile of tissues will be possible, for cells left behind at a crime scene. Should specific genes be found for schizophrenia or antisocial behaviour, an accurate picture of the criminal may be built up to help in his or her identification. Multiple genetic traits can be identified from single cells48 or those left on cigarettes. The level and pattern of nucleic-acid degradation from a range of tissues may allow a more accurate estimate of time of death than has been previously possible. Archival material stored from patients who have had detailed necropsies is also a unique resource. Many clinical studies are done on potentially biased populations of hospital patients who have survived a disease. It could be argued that the most important patients are those who succumbed quickly to a disease process, many of them in the community. These may be the most informative high-risk group to the gene hunters searching for a genetic predisposition.

DNA fingerprinting techniques are also useful in routine surgical pathology. Mix-ups of specimens occurs at any stage from the clinic to the pathologist's microscope. Suspected switches of material can be easily identified with panels of microsatellites. Such studies can prevent the wrong patient from having major surgery as well as removing the necessity of further invasive procedures to obtain more tissue. Individual biopsy samples can be microdissected and even possible extraneous material such as "floaters" can be amplified to confirm an origin from another person's surgical specimen. We now routinely offer such services. The future

Molecular pathology

How will these changes affect histopathology as a speciality? There are two main possibilities. The preferred option is that the new techniques become skills of the histopathologist so that there is a seamless speciality between morphological diagnosis and the use of genomic and proteomic data-in much the same way as immunohistochemistry is used today. This will probably not occur unless trainee pathologists are exposed to these technologies.

The other possibility is the emergence of molecular pathologists in large teaching centres to whom the material is referred. This is more probable if the technologies are expensive and highly specialised such as cDNA arrays or great advances in automation occur with the development of large-scale, rapid, high-throughput machines capable of analysing molecular lesions for a wide range of diseases.

Arrays

We are on the edge of a substantial technological revolution. The invention of large-scale mutationdetection methods by companies such as Affymetrix, and the development of expression arrays by Brown and colleagues at Stanford University, open up new fields of research. Many of the specialities mentioned would hugely benefit from mutation detection of disease,53 therapy,54 or toxicity related genes. Expression arrays will allow the simultaneous analysis of the expression pattern of thousands of genes. Thus one experiment is no longer confined to one question about the behaviour of another gene but can look at whole classes of genes: an example is the study that showed that when fibroblasts were exposed to serum, not only were cell-cycle-related genes switched on but wound-healing genes were also.55 Another example is the comparative genomics of BCG vaccines by wholegenome microarrays, by which the evolution of these vaccines over the past 90 years was shown.56 Such findings will be commonplace. To the pathologist, the disadvantage is the complexity of the tissues, composed as they are of heterogeneous tumour cells, cells of the immune response, and cells concerned with healing and repair. A great challenge will be the interpretation of the information from the arrays. Arrays can also be prepared with large numbers of cores of different tissues to look at expression of one or two markers simultaneously in multiple tumours rather than multiple markers in a single tissue or cell type.57

One note of caution for the molecular pathologist must be raised. The image of the disease that one sees under the microscope represents a summation of the genetic changes and therefore provides a lot of the key information about the disease relatively simply. Will a microarray tell us any more? We await the results from this rapidly developing area with great interest.

New methods of microscopy

Advances in light and electron microscopy have enabled substantial advances to be made in histopathology. New forms of microscopy enable the morphologist to assess DNA, proteins, and their interactions at the molecular level. Atomic-force microscopy is a form of physical microscopy that allows the shape of a structure under analysis to be visualised down to the atom (figure 1). It is possible to use this method on routine paraffin sections with or without immunohistochemistry, but its power is derived from its ability to image DNA and individual proteins (figure 2). This opens the possibility of studying normal Ad abnormal protein-DNA interactions in realtime under physiological conditions. The boundaries of the technique are unknown. Atomic-force microscopes have achieved resolution down to the atom on metals but their resolution is limited with biological material. Different forms of atomic-force microscopy include scanning near-field optical microscopy, which allows simultaneous physical and fluorescent analysis, and chemical-force microscopy, which measures the force of a chemical interaction between two biological molecules.

Conclusions

We are on the edge of a period of radical change in pathology. There will be a massive increase in our knowledge base of human disease, which will be accompanied by rapid advances in technology and allow us to make diagnostic leaps and bounds. Most histopathologists are unaware of the potential of the new biology and must embrace the opportunities. They must recognise and accept that 21 st century pathology will probably be very different to our current practice. We must evolve or face the consequences.

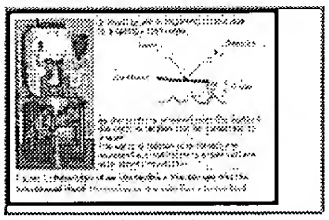


Figure 1:

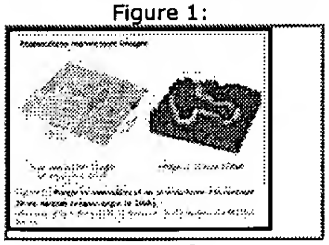


Figure 2:

[Reference]

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[Author note]

Department of Histopathology, Leeds General Infirmary, Leeds, and University of Leeds, Leeds LS2 9JT UK (Prof P Quirke MRCPATH N Mapstone MRCPATH)
Correspondence to: Prof Phil Quirke (email: philq@pathology.leeds.ac.uk)